Endocrine responsiveness in estrogen receptor-positive breast cancer

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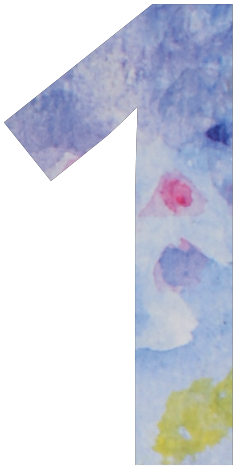
2020

document version
Publisher's PDF, also known as Version of record

Chapter



General introduction

Breast cancer epidemiology

Breast cancer is the most common cancer diagnosis for women worldwide[1]. In The Netherlands in 2016, approximately 14,000 women received the diagnosis invasive breast cancer and another 3,150 died of the disease[2], making it the second most common cause of cancer-related death. The risk of developing invasive breast cancer is slowly increasing for women in The Netherlands, from 10.5% in 1990 to 13.6% in 2010 meaning that now 1 out of 7.4 women will be affected[3].

Breast cancer survival has improved in the last decades, but the lifetime risk of dying from breast cancer remains 3.8%. Survival depends to a certain extent on tumor stage[4]. In The Netherlands, the majority of breast cancer patients are diagnosed in stage I (localized) disease (46%), while 6% present with stage IV (advanced) disease[5]. When a patient is treated according to the current guidelines, the 5-year survival rate for stage I is 98%. This percentage drops tremendously to only 29% for stage IV disease.

Apart from stage, survival is affected by breast cancer subtype. Breast cancer subtypes are commonly grouped on the basis of expression of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor type 2 (HER2) and Ki67 score[6] (Table 1). Approximately 70% of breast carcinomas are ER-positive(ER+)/HER2-negative, a subtype characterized by a relatively good prognosis compared to HER2-positive (HER2+) breast cancer or triple-negative breast cancer (TNBC).

Table 1. Breast cancer subtypes and preferred systemic treatment

Subtype	Immunohistochemical characteristics	Systemic treatment
Luminal A	ER and PR strong +, HER2-, Ki67 low	Endocrine therapy
Luminal B	ER+ or PR+, HER2-, Ki67 high	Endocrine therapy / chemotherapy
Luminal HER2-positive	HER2 overexpression or amplification, ER+ or PR+, Ki67 any	Endocrine therapy or chemotherapy without or with anti-HER2 therapy
Non-luminal HER2-positive	HER2 overexpression or amplification, ER-, PR-, Ki67 any	Chemotherapy combined with anti-HER2 therapy
Triple Negative	ER-, PR-, HER2-, Ki67 any	Chemotherapy

ER: estrogen receptor; PR: progesterone receptor; +: positive; -: negative

Since the focus of this thesis is on ER+/HER2- breast cancer, the next paragraphs will be dedicated to the current knowledge of reasons for endocrine failure, new endocrine treatment strategies and search for biomarkers that might guide relevant treatment choices.

Systemic endocrine treatment for ER+/HER2- breast cancer

Adjuvant treatment

Currently, ER+/HER2- breast cancer treatment consists of (a combination of) surgery, radiotherapy and/or systemic therapy, such as endocrine therapy with or without chemotherapy (Table 1). The type and combination of treatment largely depends on the subtype, stage and menopausal status at the time of diagnosis[7,8]. Loco-regional control is achieved by surgery or radiotherapy, but systemic therapy is indicated in patients with a high risk of developing recurrent disease from micro metastases[9]. Factors associated with the risk of recurrence are age, performance status, tumor size, tumor grade, extent of lymph node involvement, receptor status and Ki-67 proliferation score[7].

Patients diagnosed with high-risk ER+/HER2- primary breast cancer may be candidates for chemotherapy before surgery (neoadjuvant therapy) or afterwards (adjuvant therapy). Such patients and also those at lower risk will receive adjuvant endocrine therapy as well, since this has shown to improve recurrence-free and overall survival (OS)[10,11]. Recent guidelines recommend a duration of at least five years with the preferential endocrine option depending on the menopausal status[7]. For postmenopausal patients, an aromatase inhibitor (AI) e.g. letrozole, anastrozole or exemestane, or sequential treatment with the anti-estrogen tamoxifen and an AI is recommended. Tamoxifen for five years can be an alternative, if side-effects of AIs are not tolerated[11,12]. Pre/perimenopausal patients should not be treated with AI monotherapy. For these patients tamoxifen is an option. If at higher risk, ovarian suppression with an LHRH agonist or oophorectomy combined with tamoxifen or an AI for five years is recommended, since it has been demonstrated that the addition of ovarian suppression improves survival rate compared to tamoxifen alone[13,14]. Treatment duration can be prolonged up to ten years depending on poor prognostic factors[7,15].

Metastatic treatment

Some ER+/HER2 breast cancer patients may have metastatic disease at first presentation. Other patients treated for primary disease may show a recurrence which can occur even after decades[16]. When a patient develops metastases, treatment goals become palliative in nature, primarily focused on decreasing tumor

size, reducing tumor-related complaints and extending survival with preservation of quality of life. Patients with rapidly progressive visceral metastases or primary endocrine resistance have an indication for first-line palliative chemotherapy. For most postmenopausal patients, endocrine therapy forms the mainstay of treatment[11,12]. Multiple endocrine treatment regimens for metastatic breast cancer (MBC) are registered in The Netherlands as shown in Table 2[7]. First-line treatment usually consists of letrozole or anastrozole alone or in combination with a CDK4/6 inhibitor. Next, treatment with exemestane alone or in combination with everolimus, fulvestrant with or without palbociclib or abemaciclib, or tamoxifen is recommended. The optimal order of second or following lines of endocrine therapy depends on previous treatment benefit and needs to be carefully weighed for each individual patient. Premenopausal patients may receive the same drugs after addition of an LHRH agonist or after oophorectomy to induce menopause. Treatment continues until progressive disease is determined or until a patient needs to stop due to intolerable side-effects after which another line of endocrine therapy may be offered. Ultimately, disease progression will occur in all MBC patients after which palliative chemotherapy may be an option.

Table 2. Endocrine treatment for postmenopausal, ER+/HER2- metastatic breast cancer patients*

Anastrozole or letrozole without or with palbociclib or ribociclib or abemaciclib°
Fulvestrant without or with palbociclib or abemaciclib°
Exemestane without or with everolimus
Tamoxifen
Megestrol

* For pre/perimenopausal patients, an LHRH agonist/ oophorectomy needs to be added to induce menopause
° CDK4/6 inhibitor can be given in only one line of treatment

Endocrine resistance

Despite improvement in survival outcome, not all ER+/HER2- breast cancer patients benefit from endocrine therapy. Treatment efficacy is limited as a result of intrinsic or acquired resistance of tumor cells. Intrinsic or de novo resistance is characterized by ER+ breast cancer that is fundamentally irresponsive to endocrine therapy without previous treatment exposure. Acquired resistance is defined by progressive disease after initial benefit and lack of responsiveness to subsequent endocrine therapies[17].

Extensive research has been performed into oncogenic transformation causing endocrine resistance[18,19]. In a subset of tumors, resistance can be explained

by modifications of the ER receptor. For instance, breast cancer cells can lose expression of the ER receptor, although this happens in only a minority of ER+ tumors[20]. Alternatively, mutations may occur in the ligand binding domain of *ESR1* leading to hormone-independent activity of the mutated receptor[21]. The most commonly described mutations in the ligand binding domain can be found in the Y537 and D538 amino acid residues of *ESR1*[21].

Activation of cell signaling pathways may contribute to endocrine resistance, such as the mitogen-activated protein kinase (MAPK) pathway or the phosphatidylinositol 3-kinase (PI3K) – Akt – mammalian target of rapamycin (mTOR) pathway (Figure 1)[18,22] with the latter being the most frequently altered pathway in breast cancer[23]. When these pathways become activated, they can promote abnormal RNA translation, proliferation, cell growth and survival[22,24,25], processes important for tumor cell function. Moreover, both pathways interact with ER signaling through cross-talks[17,24] by which they diminish endocrine sensitivity as well. Alterations in genes encoding proteins in the PI3K pathway, like *PIK3CA* or *PTEN*, occur in over 75% of primary breast cancer cases[23]. Whether the presence of these mutations leads to endocrine resistance is not entirely clear, since in two previous studies[23,26] no correlation was found with protein activation of the corresponding pathway. Phosphorylation of Akt[27] or ERK1/2[28] as reflection of, respectively, PI3K or MAPK pathway activation has been found in breast cancer as well.

Other molecular mechanisms contributing to endocrine resistance include overexpression or amplification of growth factor receptors, like the HER2 receptor or the insulin-like growth factor-1 receptor β (IGF-1R)[22,29]. Once activated, these growth factor receptors can activate downstream signaling of the PI3K and MAPK pathways and, as explained above, promote tumor cell growth and survival. Cell line studies show that phosphorylation of the IGF-1R receptor and concurrent activation of the PI3K and/or MAPK pathway is able to drive endocrine resistance[30-32], but this phenomenon has not yet not been studied in a clinical dataset.

Tumor cells may gain resistance through dysregulation of cell cycle checkpoints. One well recognized mechanism is associated with the retinoblastoma (RB) protein and the cyclin D1-cyclin-dependent kinases (CDK)4/6 complex[33,34]. In normal cells, RB inhibits cell proliferation at the G1 checkpoint during mitosis. Under the influence of pro-mitotic signals, the CDK4/6 complex phosphorylates RB, allowing the cell to continue the cell cycle and proliferate. Tumor cells have been shown to upregulate parts of the cyclin D1-CDK4/6 complex[23], avoiding anti-mitotic signaling and as a result will proliferate despite endocrine treatment.

Regardless of the specific mechanisms, all cause ER+ breast cancer cells to be less or irresponsive to ER-targeted treatments. Notably, one must always keep in

mind that breast cancer is a heterogeneous and adjustable disease. Among patients and also within the same patient, multiple resistance mechanisms can co-exist or subsequently develop, making it difficult to identify one mechanism as causal factor.

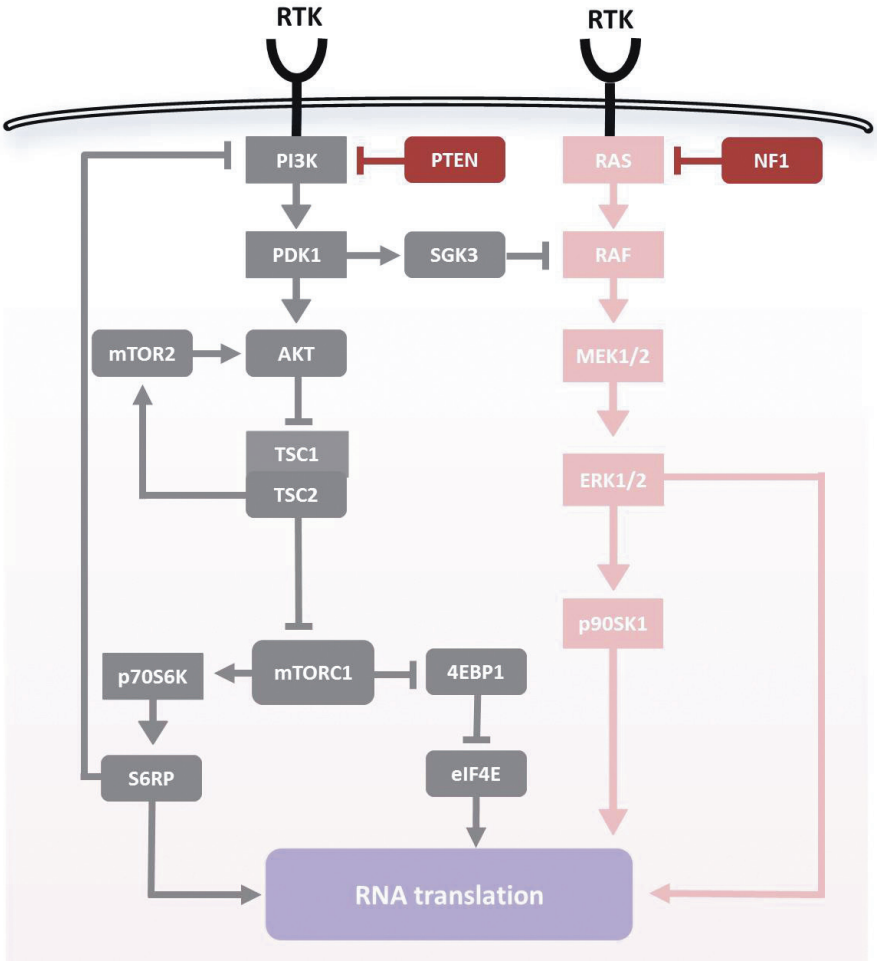


Figure 1. Phosphatidylinositol 3-kinase (PI3K) (grey) and mitogen-activated protein kinase (MAPK) (pink) signaling network, showing examples of cross-talk and feedback loops. Both pathways function downstream of receptor tyrosine kinases (RTKs) and G protein-coupled receptors. Mammalian target of rapamycin (mTOR) complex 1 (mTORC1); tuberous sclerosis (TSC) complex (TSC1 combined with TSC2); p70 ribosomal protein S6 kinase (p70S6K); eukaryotic translation initiation factor eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4EBP1); 40S ribosomal protein S6 (S6RP); phosphatase and tensin homolog (PTEN); mitogen-activated and extracellular signal-regulated kinase kinase (MEK)1/2; extracellular signal-regulated kinase (ERK)1/2; p90 ribosomal six kinase-1 (p90RSK1); neurofibromatosis type 1 (NF1); phosphoinositide-dependent protein kinase 1 (PDK1); serum- and glucocorticoid-regulated kinase 3 (SGK3). (Figure partly adapted from Kruger et al, 2018[35])

New treatment strategies to overcome endocrine resistance

Exemestane plus everolimus

With the discovery of various resistance mechanisms, new targeted agents are being developed to overcome endocrine resistance. One of them is everolimus, a rapamycin analogue which inhibits the mammalian target of rapamycin-containing complex 1 (mTORC1), a key component in the PI3K pathway (Figure 1). Blocking mTORC1 may prevent tumor growth caused by activation of the pathway. The efficacy of everolimus was demonstrated in the BOLERO-2 study, a double-blind, randomized, placebo-controlled clinical trial[36]. In that study, patients with ER+/HER2- MBC refractory to a non-steroidal aromatase inhibitor (NSAI) who received everolimus combined with exemestane had an improved progression-free survival (PFS) compared to those receiving exemestane plus placebo (7.8 vs 4.1 months, respectively). This has led to the registration of everolimus with exemestane (EVE/EXE) treatment for patients with ER+/HER2- MBC refractory to a NSAI. The improved PFS found in the BOLERO-2 study was confirmed in subsequent open-label trials on this combination in MBC[37-40].

Nowadays, MBC patients refractory to an NSAI may receive standard everolimus 10 mg and exemestane 25 mg orally per day. Frail patients can start with a dose of 5 mg daily for everolimus, but in the absence of symptoms it is advised to increase the dose to 10 mg after two weeks. However, some patients suffer from side-effects that require dose reduction, interruption or discontinuation. In the case of adverse events (AEs) suspected to be related to everolimus, it can be temporarily interrupted or its dose can be reduced to 5 mg daily with a minimum of least 2.5 mg daily, while exemestane should be continued in all cases. The most common AEs in the BOLERO-2 study leading to dose reductions or interruptions due to everolimus were stomatitis, pneumonitis, alanine aminotransferase increase, aspartate aminotransferase increase, dyspnea, blood creatinine increase and fatigue[41].

Unfortunately, a proportion of patients needlessly suffers from side-effects since they do not benefit from the combination that also comes with considerable costs. Therefore, there is a high need for markers that select patients who will likely benefit from EVE/EXE or, the reverse, withhold treatment from patients with resistant disease.

Endocrine therapy + CDK4/6 inhibitors

Besides inhibition of the PI3K pathway by everolimus, efforts have been made to tackle other resistance mechanisms. One of the major breakthroughs is the development of CDK4/6 inhibitors which interact within the cell cycle and thereby

prevent proliferation[42,43]. Three CDK4/6 inhibitors palbociclib, ribociclib and abemaciclib in combination with endocrine therapy are already in clinical use. Clinical trials are ongoing in different disease situations and follow-up studies are carried out to analyze whether these drugs not only improve PFS, but also OS.

In the double-blind, randomized PALOMA-2 study, first-line letrozole plus palbociclib has been compared with letrozole plus placebo in postmenopausal women with ER+/HER2- breast cancer[43]. An improvement in PFS was demonstrated from 14.5 months in the letrozole plus placebo group to 24.8 months in the group treated with palbociclib plus letrozole. Also, when given as second-line treatment in combination with fulvestrant, the addition of palbociclib remained favorable. In the PALOMA-3 study, patients who received palbociclib plus fulvestrant had a median PFS of 9.5 months which was significantly longer than the 4.6 months in the fulvestrant plus placebo group[44].

The MONALEESA-2 study has demonstrated the benefit of letrozole combined with ribociclib as first-line treatment for MBC patients. The combination showed a PFS of 25.3 months, while 16.0 months was reached for placebo plus letrozole[45]. The MONALEESA-3 study has shown the efficacy of ribociclib plus fulvestrant as first or following line of treatment for ER+/HER2- MBC patients[46]. However, ribociclib as second line treatment is not covered by the health insurances in The Netherlands at the moment.

Very recently, the CDK4/6 inhibitor abemaciclib is now available for ER+/HER2- MBC patients. A PFS benefit of abemaciclib in combination with anastrozole or letrozole compared to placebo with an NSAI as the first-line treatment was demonstrated in the MONARCH 3 study (HR 0.54, $p = 0.000021$)[47]. In another study with abemaciclib, the MONARCH 2[48], abemaciclib plus fulvestrant significantly extended PFS compared to fulvestrant plus placebo in women with HR+/HER2- MBC who had progressed on previous endocrine therapy.

Side-effects of CDK4/6 inhibitors are group dependent and therefore overlap[43-48]. The most common grade 3 or 4 adverse events were myelosuppression for all three drugs, fatigue for palbociclib and abemaciclib, while diarrhea and nausea were more common in abemaciclib-treated patients.

Biomarkers in breast cancer

Research has focused on the identification of predictive and prognostic biomarkers that might be relevant for patient selection or therapy guidance in breast cancer[49,50]. Prognostic biomarkers may be used to estimate the natural course of the disease irrespective of treatment, while predictive biomarkers might point towards benefit of specific treatments[51]. In other words, prognostic biomarkers are useful to discern

high-risk patients who need extra treatment to prevent metastatic disease and predictive markers are valuable to select the best treatment for the right patient. To be able to discriminate between prognostic and predictive markers, comparative studies in patient groups receiving a particular treatment or not is essential[51], but potential biomarker material, such as blood or tumor tissue, from patients who participated in such studies is scarce. Importantly, the possible benefits for (future) patients must outweigh any possible harm caused by the biomarker test.

Since activation of the PI3K and MAPK pathways is associated with a higher chance to develop resistance to anti-estrogens or estrogen deprivation, efforts have been made to find predictive and prognostic biomarkers associated with pathway activation. With the use of immunohistochemistry (IHC) both total levels and phosphorylation of proteins downstream in the pathways have been studied. High levels of phosphorylated p70S6K (p-p70S6K) in primary breast cancer patients were associated with less adjuvant tamoxifen benefit[52]. Similarly, tamoxifen was less effective in patients with tumors that expressed high p-mTOR and positive p-ERK1/2[52]. In two different studies within the same population, high expression of S6K1[53] or strong cytoplasmic p-4EBP1[54] predicted a worse prognosis, while strong nuclear 4EBP1 correlated with good prognosis[54]. The prognostic significance of *PIK3CA* mutations was analyzed in a recent meta-analysis[55]. *PIK3CA* mutation status was not associated with relapse-free or OS in ER+ breast cancer patients. In another study, *PIK3CA* mutation status was not predictive for tamoxifen benefit[26]. Notably, due to the various feedback loops and cross-talks between cell signaling pathways, a single marker is at risk to produce false-positive or false-negative results when used as readout for pathway activation. Unfortunately, besides ER, PR and HER2, none of the described markers has made it into clinical practice.

Efforts have already been made to find predictive markers for MBC patients who received everolimus combined with endocrine therapy. Analyses of *PIK3CA*, *FGFR1*, and *CCND1* mutations in tumour tissue as well as *PIK3CA* mutations in cell-free DNA in plasma have been carried out in patients who participated in the BOLERO-2 study [56,57]. In these studies, median PFS on the combination everolimus plus exemestane was maintained regardless of mutated or wild type status. Yi et al[58] have analyzed mutations in ctDNA of 16 ER-positive breast cancer patients treated with everolimus and reported that patients with a H1047R mutation in *PIK3CA* had a longer PFS than patients with wild-type *PIK3CA.* Another group has used a Mass Array Sequenom platform to analyze the genetic status in 25 archival tumour specimens of breast cancer patients treated with everolimus and exemestane for advanced disease[59]. They found that the median PFS was shorter in patients with detected mutations compared to those without mutations. Immunohistochemistry

(IHC) analyses in primary tumour tissue of patients in the TAMRAD study has suggested possible associations between high levels of p-4EBP1, low 4EBP1, low LKB1 or low p-Akt(Ser473) levels and improved time-to-progression from everolimus plus tamoxifen, but tissue from only few patients could be obtained[60]. In two small studies of patients treated with everolimus and exemestane, no association between PTEN expression[61,62] or p-S6RP(Ser235/236) staining scores[61] and PFS was detected. Taken together, a suitable marker to select patients likely to benefit from everolimus with exemestane is not yet available.

Outline of this thesis

In this thesis, we aimed to investigate potential biomarkers for primary and metastatic ER+ breast cancer that are either prognostic for the course of the disease or predictive for the success of endocrine treatment. **Chapter 1** gives a general introduction of this thesis.

Part I of this thesis is focused on finding putative markers within the PI3K and MAPK pathways in a historical cohort of primary ER+ breast cancer patients randomized to either adjuvant tamoxifen or placebo. Since various feedback loops and cross-talks between these pathways exist, a single marker is at risk to produce false-positive or false-negative results when used as readout for pathway activation. We, therefore, searched for a better readout of PI3K and MAPK pathway activation in ER+/ HER2– breast cancer patients that might be predictive for endocrine resistance or associated with prognosis as described in **Chapter 2**. We performed IHC of multiple (phosphorylated) proteins followed by unsupervised hierarchical clustering to show for the first time how seven proteins downstream in both pathways are expressed in ER+/HER2- breast cancer cases and linked differences in pathway expression with prognosis and prediction of the efficacy of tamoxifen. A potential tool to predict the usefulness of adjuvant tamoxifen was also developed. In **Chapter 3**, unsupervised hierarchical clustering, similar to the technique described in Chapter 2, was performed to show how seven proteins of the PI3K and MAPK pathways cluster in ER+/HER2-positive, ER-negative/HER2-positive and in TNBC cases, since this had not yet been explored in these BC subtypes. In **Chapter 4**, the predictive value of positive IGF-1R expression for the outcome of adjuvant tamoxifen was studied. In previous research, cell line studies have shown that activation of the IGF-1R pathway is able to drive endocrine resistance, but this phenomenon had not yet been investigated in a clinical dataset. We studied whether positive p-IGF-1R/InsR expression diminished adjuvant tamoxifen benefit in a large cohort of primary ER+/IGF-1R+ breast cancer patients. Furthermore, we searched for

approaches to overcome this IGF-1R-mediated tamoxifen failure in breast cancer cell lines and showed the potential usefulness of the dual IGF-1R/InsR inhibitor linsitinib to overcome tamoxifen resistance in IGF-1R-driven ER+ breast cancer.

Part II focusses on possible biomarkers to select patients who have a high chance for a long PFS, while using standard EVE/EXE. This was carried out in the context of an exploratory, open-label, single arm, multicenter study: the Everolimus Biomarker Study (ClinicalTrials.gov Identifier: NCT02109913; EudraCT number 2013-004120-11). In this study, blood and primary tumor tissue was received from 175 postmenopausal women with ER+/HER2- MBC, refractory to an NSAI and who received standard EVE/EXE. 28 patients gave informed consent for an additional biopsy of a reachable tumor location. In **Chapter 5** blood samples were analyzed with Next Generation Sequencing (NGS) with molecular barcoding which enabled us to detect tumor-derived mutations in cell-free DNA (cfDNA) from plasma of breast cancer patients without the need of invasive biopsies[63]. This promising less invasive technique was used to assess the occurrence of 10 most commonly affected genes derived from circulating tumor DNA (ctDNA) and their possible association with PFS and OS. As such, this technique might be useful to select MBC patients with possible benefit from EVE/EXE. In **Chapter 6** we made an attempt to find a possible biomarker in tumor tissue by IHC for multiple (phosphorylated) proteins of the PI3K/MAPK pathway that might indicate potential benefit from EVE/EXE. We analyzed these proteins in primary tumor tissue and in a number of new tumor biopsies before the start of EVE/EXE and linked these results with PFS. Finally, the various study results described in this thesis are summarized and discussed in **Chapter 7**.

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